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13. ABSTRACT (Maximum 200 Words) Oxidative stress may play a role in human oncogenesis, including breast cancer. The mitochondria are most common sources of reactive oxygen species (ROS) responsible for most oxidative stress. This project evaluates the role of mitochondrial abnormalities in oxidative stress in breast cancer development. Transgenic mice harboring mutant mitochondrial Complex II subunit targeted in the mammary glands will be characterized in terms of mitochondrial functions, ROS production and oncogenesis. The effects of oxidative stress in other transgenic mouse models of breast cancer or predisposed mice will be generated by cross-breeding and analyzed in terms of their courses of oncogenesis in the presence or absence of the mitochondrial mutant transgene, and hence oxidative stress. This study should provide significant information regarding the role of oxidative stress in breast cancer development and progression, and insights on whether antioxidants are beneficial in prevention and treatment of such important cancer in women.				
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INTRODUCTION

Oxidative stress has been postulated to contribute to numerous human diseases, including aging, neurodegeneration, cardiovascular disease, and cancer (1,2). Since most studies were conducted at epidemiological levels, the experimental proof for such a causative effect has been lacking. The overall goal of this project is to address this question in transgenic mouse modeling focusing on breast cancer.

Numerous mutations with various mitochondrial components have been demonstrated to cause increase in reactive oxygen species (ROS), the ultimate mediators of oxidative stress. In particular, mutations in the subunits of the mitochondrial Complex II have been demonstrated to produce structural abnormalities, impair energy production, electron transport, and generate ROS (3,5,9). To address the role of mitochondrial structural abnormalities and ROS in breast cancer development and progression, an advanced strategy has been implemented to over-express a subunit of the mitochondrial Complex II in the mammary glands of transgenic mice. The effects of such mitochondrial abnormalities and elevation of oxidative stress will be evaluated in these animals in terms of breast cancer development under normal and predisposed conditions. The first two years of this project have been devoted to generating the transgenic mice and establishing the transgenic mouse models of breast cancer.

BODY

Task 1. To construct and characterize mutant transgenic mice

The Cre-LoxP Transgene Activation Strategy

As illustrated both in the original application and the first annual report, the Cre-LoxP transgene activation (6-8) was initially adopted for the present study to target the expression of a mutant Complex II subunit in the mammary glands of transgenic mice. This system consists of two components, an activator that harbors a Cre recombinase transgene directed by a mammary gland specific promoter, such as the whey acid protein (WAP) or MMTV promoter, and a responder that harbors a bicistronic transgene directed by a strong actin promoter. Independently, these two transgenes will not produce any mutant Complex II subunits. However, when they are present in the same transgenic mice, the expression of the Cre recombinase will recombine the responder gene, thereby placing the Complex II mutant directly under the regulation of the strong actin promoter, resulting from a switch of transgene expression from the β -galactosidase to mutant Complex II subunit and an indicator molecule, the green fluorescent protein (GFP).

As reported in the first annual report, we have generated all the mutant Complex II subunit genes, i.e. V69E on subunit C and H102L in subunit D using site-directed mutagenesis. They were inserted into the responder vector, PCCALL2-IRES-EGFP (4), and used for transgenic mouse construction. For the mSdhC-V69E mutant, we have generated a total of 16 founder animals. To identify those with functional transgenes, we analyzed the expression of the β -galactosidase (= lacZ) gene and identified 3 transgenic lines that express the transgene. For the mSdhD-H102L Complex II mutant, we obtained 6 founder animals. These transgenic lines were then crossed with non-transgenic mice to establish the respective transgenic lines.

For the activator lines, we had obtained transgenic lines, WAP-Cre (7) and CAG-Cre (6) from the Jackson Laboratory and the Mouse Models of Human Cancer Consortium at the National Cancer Institute through the Material Transfer Agreement Process. Due to the patent held by the Dupont Corporation on the Cre-LoxP system, negotiation was conducted by the Regional Counsel Office at the Veterans Affairs Medical Center, San Francisco. The entire process took approximately 4 months, after which breeder pairs of the respective transgenic mouse lines were transferred to the Animal Care Facility at the VA Medical Center, San Francisco. They were finally integrated into the breeding facilities after a quarantine period of two months at the isolated quarter.

Task 2. To correlate Complex II mutant expression to ROS synthesis and mammary oncogenesis in transgenic mice

The availability of the various activator and responder transgenic line now allows us to proceed with the assembly of the transgene activation system by crossing these transgenic lines to generate bi-transgenic mice. To facilitate such study, we initiated a breeding program to establish breeding colonies for both WAP-Cre and CAG-Cre mice. Once sufficient animals are available, they will be used in crossing experiments to the appropriate responder lines, harboring the mSdhC-V69E and mSdhD-H102L mutant Complex II transgenes.

To test whether any of the responder lines are capable of responding to a Cre mediated recombination, we have initiated crossing of breeding mice from Jackson Laboratory and the Mouse Cancer Model repository at NCI, particularly the CAG-Cre and another transgenic line, TSPY-Cre, generated in our own laboratory. The objective of these preliminary studies is to identify those functional responder lines for experiments to be conducted at a later stage with the mammary gland specific Cre recombinase lines. These experiments have just begun and, so far, we have obtained 2 double transgenic mice harboring both TSPY-Cre and mSdhD-H102L transgenes. The recombination status of their responder gene is currently being investigated.

Low Levels of Mutant Complex II Transgene Expression in Founder Animals

As discussed above and in previous annual report, we have generated 16 founders for the floxed mSdhC-V69E and 5 founders for the floxed mSdhD-H102L responder constructs. Of these, only a selected number of founder animals were capable of transmitting their transgenes to their offspring. Most transgenic founders are in the FVB genetic strain background. As these founders aged, we observed a series of abnormalities among those harboring the mSdhD-H102L construct, but not those harboring the mSdhC-V69E transgene or 6 additional founder animals harboring another transgene, TSPY, generated in our laboratory on another project. We surmise that these abnormalities might be related to low levels of the mutant mSdhD-H102L transgene expression, due to the leakiness of the gene construct. The PCCALL2-mSdhD-H102L-IRES-EGFP expression vector (Figure 1) is directed by a strong actin promoter that is ubiquitously active in most cell types.

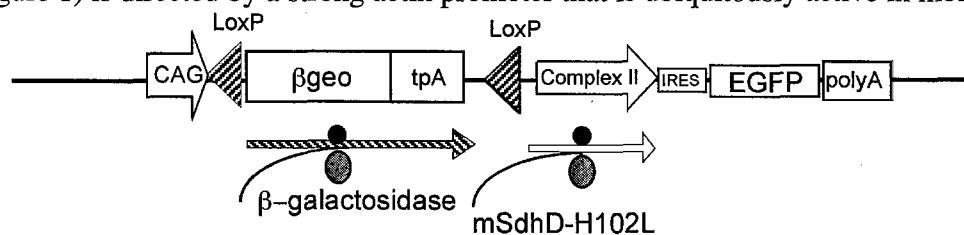


Figure 1. PCCALL2-mSdhD-H102L-IRES-EGFP transgene construct

Such high levels of tri-cistronic transcripts from this transgene might allow a low level of translational initiation at the coding sequence for the mSdhD-H102L, thereby expressing low levels of this mutant Complex II subunit. We postulate that a chronic expression of this Complex II mutant might have resulted in unintended consequences manifested phenotypically.

The most prominent abnormality is the development of benign tumor-like mass on the flanks of 3 founding animals harboring the mSdhD-H102L transgene (Figure 2) at 8-12 months of age. Dissection of the tumors showed that they contained fatty masses, resembling tissue vascularization. Such tumor-mass disappeared when the transgene of one (#5 founder) of these 3 founders was introduced into a CD-1 background, suggesting that the FVB genetic background is important for its manifestation. The transgenes in the remainder two founders could not be transmitted to their offspring.

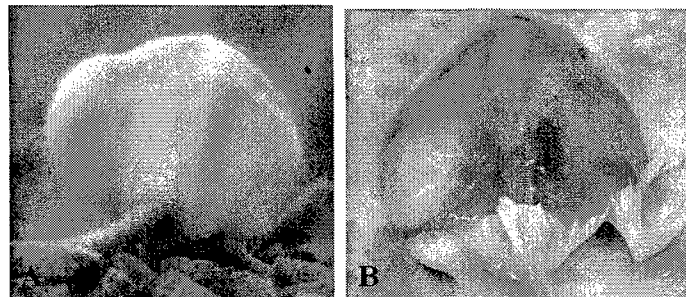


Figure 2. Tumor-like mass on the flanks of mSdhD-H102L responder transgenic founder.

Another mSdhD-H102L founder developed asthma-like breathing disorder and was sacrificed to release its suffering at 14 months of age. Necropsy revealed an abnormality on the right lung of this animal (Figure 3). Histologically, the abnormal lung lobe showed cancerous lesions that were absent in the normal lobe.

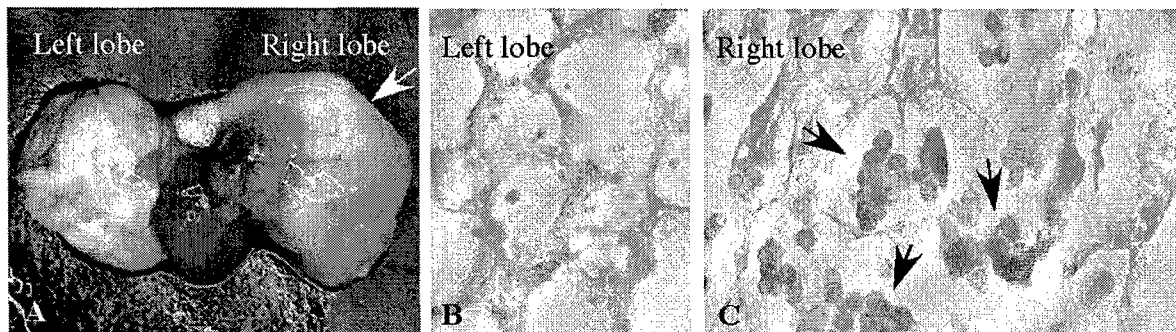


Figure 3. Lung lesions in a mSdhD-H102L founder mouse. A) Gross morphology of the lung lobes; B-C) Histology of lung sections from left (normal) and right (cancerous) lobe respectively. Arrows indicate the cancerous lesions.

Refinement of the Transgenic Mouse Strategy

The high frequency of abnormalities among the founders of mSdhD-H102L transgene suggests that a leaky expression of this mutant Complex II, abide at extremely low levels, could have effects on the host animals. To seek confirmation on such postulation, we need to address the

possibility of the mutation H102L in the mSdhD subunit of Complex II could be mutagenic in these processes. Further, we plan to determine if a chronic expression of this mutant subunit disrupts the Complex II structure and functions, thereby elucidating a certain level of oxidative stress in the affected tissues. Ultimately, we like to target such mutant to the mammary glands of the transgenic mice and evaluate its effects on breast cancer development, the initial goal of this Idea project.

Currently, we believe that the Cre-LoxP transgene activation strategy (6-8) should still be a definitive study to establish the role of mutant subunit Complex II, particular the mSdhD-H102L mutation, in the oncogenic process using transgenic mouse technique. However, given the sophistication of the strategy and the time-consuming nature of transgenic mouse breeding and operation, this objective might not be achievable within the time frame of this project. To quickly address the importance of the mSdhD-H102L mutation in Complex II structure and function, and its relationship to oxidative stress and oncogenesis in the mammary glands, we plan to simplify our transgenic mouse strategy and to target the expression of such a mutant subunit in the mammary glands by a tissue-specific promoter, the whey acidic protein (WAP). We surmise that WAP promoter has been used extensively and successfully in targeting the expression of other oncogenic genes in the mammary glands of transgenic mice that eventually develop breast cancer. Since generation of oxidative stress in the mammary glands might not be detrimental to the host animals, we should be able to observe the effects due the expression of the mSdhD-H102L mutant in breast cancer development and progression.

To accomplish this goal, we have obtained a WAP-expression cassette consisting of 2.42-kb of WAP gene 5'-flanking sequence and 4.6-kb of WAP-3'-flanking sequence from Dr. Eric Sandgren via Dr. Jeffrey Ross, University of Wisconsin. Previous studies have demonstrated that insertion of a mini-gene in between these two WAP gene sequences resulted in high expression of the inserted gene in the mammary glands of the transgenic mice (10,11). To generate a mSdhD expression cassette, we inserted either the mSdhD-H102L or the wild type coding sequence, followed by an IRES (internal ribosome entry site) and the reporter gene, EGFP, between these WAP 5' and 3' flanking sequences (Figure 4). Both the wild type and the normal and mutant mSdhD subunits are tagged with the V5 epitope, so that the products of the transgenes can be distinguished efficiently from that of the host endogenous gene. Again, the IRES and EGFP sequences will provide a means to observe the transgene expression by detecting the EGFP fluorescence without sacrificing the host animals. We are currently generating transgenic mice harboring this mutant mSdhD-H102L construct.

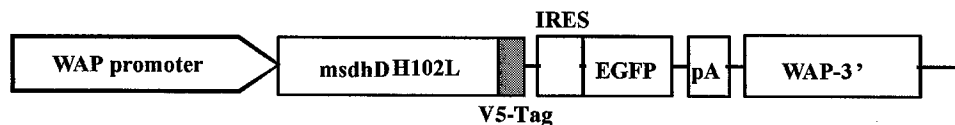


Figure 4. WAP-promoter directed mSdhD-H102L mutant construct targeting mammary gland transgene expression. A V5-epitope tag is fused with the mSdhD gene to distinguish the products between the transgene and the endogenous mSdhD gene. EGFP is included as a reporter for visualization of transgene expression in live animals.

To test the feasibility of using a small V5-tag as a means to detect transgene expression in the mitochondria, the mSdhD-H102L-V5 mutant and wild type-V5 cDNAs were expressed in COS7 cells under the CMV promoter using transient transfection technique. The transfected mSdhD products were detected by immunofluorescence (red) while the mitochondria were visualized by

MitoTracker green procedures. Our results demonstrated that the V5 is efficient in tagging the recombinant products from transfected mSdhD genes using immunofluorescence and that the mSdhD protein is co-localized in the mitochondria of the transfected cells (Figure 5).

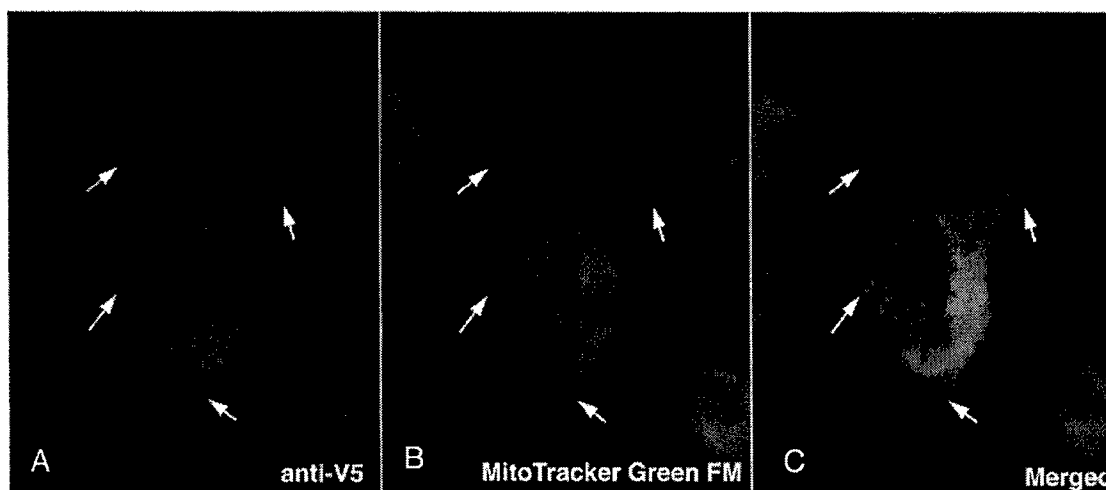


Figure 5. Expression of V5-tagged mSdhD-H102L in COS7 cells. A) Detection of mSdhD-H102L by immunofluorescence; B) Localization of mitochondria by MitoTracker Green dye; C) Merged image of A and B, indicating co-localization of mSdhD-H102L in the mitochondria (e.g. arrows).

FUTURE DIRECTIONS

We obtained preliminary results in the transgenic mice harboring a mutant Complex II subunit, mSdhD-H102L, suggesting that this mutation might be involved in tissue vascularization and/or oncogenesis in host animals. Hence, we plan to focus on this particular mutation in our studies in the coming year. To expedite our investigation, we have refined our transgenic mouse strategy to a more simplified one, targeting the expression of the mutant Complex II by mammary specific promoter in the mammary glands of the transgenic mice. This improved approach allows a more ready evaluation on the effects of mutant subunit in mammary oncogenesis. However, we believe that the Cre-LoxP gene activation system still a significant strategy in the future, and probably might need to be used beyond the one-year period remaining in this project. In any events, we consider the establishment of a breast cancer model involving the expression of a mutant Complex II subunit to be a significant step towards the evaluation of mitochondrial structural abnormalities and dysfunction and oxidative stress in this human disease.

KEY RESEARCH ACCOMPLISHMENTS

- Construction of transgenic mouse lines harboring mutant Complex II mutant expression cassette
- Acquisition of mammary gland-specific Cre recombinase transgenic mice
- Characterization of founder animals harboring mutant Complex II mSdhD-H102L transgene, demonstrating the potential involvement of this mutant gene in tissue vascularization and oncogenic lesions
- Refinement and simplification of the transgenic strategy to demonstrate the significance of the mSdhD-H102L mutation in mammary oncogenesis
- Demonstration of the feasibility of epitope-tagged mSdhD gene in transfected cells

REPORTING OUTCOMES

None.

CONCLUSION

We have made good progress in establishing the components essential for the studies proposed in the original application. We have demonstrated the potential significance of a mutant Complex II subunit, mSdhD-H102L, in tissue vascularization and oncogenesis. We have now simplified our transgenic approach specifically designed to obtain some useful results, within the time frame of this project, to indicate the likely involvement of mitochondrial abnormalities and dysfunction in oxidative stress and mammary oncogenesis.

SO WHAT

Oxidative stress has been implicated in the etiologies of numerous human diseases. Successful implementation of the proposed research will provide critical insights on its role(s) in breast cancer. The availability of experimental animal models of breast cancer, pertaining to mitochondrial structural abnormalities and oxidative stress, will be important in understanding the disease mechanisms, potential prevention and therapeutic intervention for this devastating human cancer.

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